The genetics of *Luperina nickerlii* Freyer, 1845 in Europe (Noctuidae)

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Abstract. We use mitochondrial markers to examine the genetic status of European subpopulations of *Luperina nickerlii* Freyer, 1845 (Noctuidae) in Britain, Ireland, Spain and the Czech Republic. We show that all the populations sampled belong to the same species *Luperina nickerlii*, despite considerable differences in appearance, ecology and population isolation. Neighbour-joining tree based on mitochondrial markers showed only three populations as separate clusters: *gueneei*, *nickerlii* and *knilli*. We show that subspecies *leechi*, *albarracina* and *demuthi* are genetically close to each other and that both *leechi* and *gueneei* show significantly lower heterozygosity than the other subspecies sampled. *L. n. albarracina* and *knilli* show high genetic variability. Isolation by distance was not supported in this study, suggesting populations were probably linked to each other in the recent past.

Introduction

Luperina nickerlii Freyer, 1845 (Noctuidae) is widespread in mainland Europe occurring on xerothermic slopes where the larvae feed on different grasses (Ganev 1982; Hacker 1989; Karsholt & Razowski 1996; Robineau 2007; Steiner & Ebert 1998), although in Britain and Ireland they are entirely coastal and certain subspecies are of conservation concern (Goater & Skinner 1995). Eight subspecies of this moth have been described (Tab. 1), based largely on phenotypes such as wing colouration, although the taxonomy of the genus is in constant flux. Thus, *L. n. leechi* was described as a new subspecies in 1976 (Goater 1976), whereas *L. n. graslini* and *L. n. tardenota* were originally described as separate species but have since been synonymised and are now regarded as subspecies of *L. nickerlii* (Zilli et al. 2005). *L. n. knilli* has also been proposed to warrant full species status (De Worms 1978; Haggett 1980) but is now considered a subspecies (Skinner 2008). *L. n. albarracina* was described as a new subspecies in 1962 (von Zerny 1962) on wing colour and shape, but some authorities now consider it merely a form (Zilli et al. 2005). *L. n. graslini* is similar in appearance to *L. n. gueneei* but otherwise the subspecies all look different from each other in wing colour and are generally easy to distinguish by eye. *L. n. demuthi* is the most variable of all the subspecies and occasional specimens may look similar to those of subspecies *L. n. leechi*, *L. n. knilli* and *L. n. gueneei*, but the majority of specimens are readily separated from the other subspecies.

The European mainland subspecies all have similar ecologies, feeding on the same food plants and occupying similar biotopes. However, the subspecies in Britain show dramatic differences in their ecology and life styles and are prone to producing small

isolated colonies (Tab. 1). In Britain at least, the sparse fragmented distribution of this moth over a large area may reflect a collapse in a former larger range size following an initial increase at the end of the last glacial maximum when temperatures attained levels similar to those of today, and as a result leaving behind small refuge populations, or alternatively multiple post-glacial colonisation events from continental populations or recent changes in habitat preference or behaviour (e.g., a host-plant switch or a reluctance to fly). The populations of the British and Irish subspecies are at least 300 km apart from each other and a minimum of 320 km from the nearest known mainland population near Paris (which is very small and possibly endangered), 850 km from Spanish populations and 950 km from populations at Prague, with little possibility of regular interchange. However, in captivity, *L. n. gueneei* and *L. n. leechi* (which have similar ecologies) can pair and produce moths similar to *L. n. leechi* (Haggett 1980; A. Myers, personal communication). These, at least, are the same species (Mayr 1942) and have similar ecologies. The definition of a subspecies (Lincoln et al. 1985) as isolated natural populations differing taxonomically and genetically from other groups within the species supports the splitting of *L. nickerlii* into subspecies as differing taxonomically.

The single isolated population of *L. n. leechi* has perhaps been studied in more detail than the other subspecies (e.g., Spalding 1991a, b). It is restricted to a small area of a shingle beach, 500 metres × 240 metres, where its larval food plant *Elytrigia juncea* (L.) Nevski (Poaceae) occurs (Spalding 1997; Spalding et al. 2012). It is listed in a Biodiversity Action Plan listing as a unique subspecies currently under threat and declining, and is thus of high conservation value (JNCC 2007). The population has been studied by one of us (AS) since 1987. The population size of this iconic species is very small, with the annual Index of Abundance (the sum of the weekly means based on transect counts) between 1994 and 2009 ranging between 5 to 78 adult moths (mean 18.99) (Spalding 1997; Spalding & Young 2011a).

Here we use mitochondrial DNA markers to ask several questions fundamental to the origin and conservation of *L. nickerlii* subspecies. First, given the different ecologies and appearance of the UK and Irish subspecies, do they belong to the same species as the mainland populations? Secondly, we investigate the genetic variation of these subspecies. Thirdly, we ask which populations should be the focus of conservation concern.

Material and Methods

Sampling. Adult specimens of *Luperina nickerlii* from four different populations in UK and Ireland and four mainland populations from the Czech Republic and Spain were sampled (Tab. 2 and Fig. 1). Actinic light traps with chloroform were used to collect the moths except for *L. n. leechi*, which was collected using torch light. Specimens were kept alive until either snap frozen in liquid nitrogen (*L. n. leechi*, *L. n. gueneei*, *L. n. knilli* and *L. n. demuthi*) or preserved in pure ethanol (Czech populations of *L. nickerlii*) for genomic DNA extraction except for pinned and dried specimens (Spanish populations of *L. n. albarracina*).

Tab. 1. *Luperina nickerlii* subspecies in Europe and their food plants and habitat

Subspecies	Habitat	Food plant	Countries	Number of sites	Within site abundance
demuthi Goater & Skinner, 1995	Saltmarsh	*Puccinellia maritima* (Huds.) Parl.	England	Few	Abundant
graslini Oberthür, 1908	Hot dry slopes	*Festuca ovina* L. and other grasses	France (south)	Many	Abundant
gueneei Doubleday, 1864	Sand dune	*Elytrigia juncea* (Viviani) Runemark ex Melderis	England; Wales	Few	Abundant
knilli Boursin, 1964	Coastal cliff	*Festuca rubra* L.	Ireland	Few	Rare
leechi Goater, 1976	Shingle beach	*Elytrigia juncea* (Viviani) Runemark ex Melderis	England	One	Rare
nickerlii Freyer, 1845	Sparse open grassland; sandy heaths	*Festuca ovina* L. and other grasses	Germany; Czech Republic; Bulgaria	Many	Abundant
tardenota Joannis, 1925	Hot dry slopes	*Festuca ovina* L.and other grasses	France (central)	Few	Rare
albarracina Schwingenschuss, 1962	Hot dry slopes	*Festuca ovina* L. and other grasses	Spain; Portugal	Many	Abundant

Tab. 2. *Luperina nickerlii* sampling locations and mitochondrial COI haplotypes

Date	Subspecies	Locality	Coordinates	COI*	Sample size**
11–14 Sep 2008	*leechi*	Loe Bar, UK	50°04′12.98″ N 5°17′40.14″ W	A	22
8 Sep 2008	*demuthi*	Strood, UK	51°47′51.53″ N 0°55′09.23″ E	A, B, C	10
27–28 Aug 2008	*gueneei*	Gronant, UK	53°21′01.30″ N 3°22′20.77″ W	D	12
19 Aug 2008	*knilli*	Inch, Ireland	52°08′37.41″ N 9°59′14.65″ W	A, F, G	4
19–20 Aug 2008		Trabeg, Ireland	52°07′16.44″ N 10°12′25.82″ W	E	10
5 Sep 2009	*nickerlii*	Máslovice, CR	50°12′23.80″ N 14°23′16.85″ E	H, I, J	6
5 Sep 2009		Praha, CR	50°02′53.11″ N 14°24′16.68″ E	H, I	14
15 Sep 2009	*albarracina*	Amavida, Spain	40°33′38.97″ N 5°5′7.13″ W	A, K, O	4
16 Sep 2009		Páramos, Spain	42°35′17.95″ N 3°43′56.36″ W	A, K, L, M, N	9

* cytochrome c oxidase subunit I gene (COI) haplotypes sampled in the *L. nickerlii* populations.
** Number of specimens collected.

Genomic DNA isolation. Genomic DNA was extracted from the thorax or abdomen of preserved individuals using either standard phenol/chloroform method (Blin & Stafford 1976) or a Genomic DNA Purification Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. DNA concentration was estimated by NanoVue (GE Healthcare, Buckinghamshire, UK) and adjusted to 500 ng/μl.

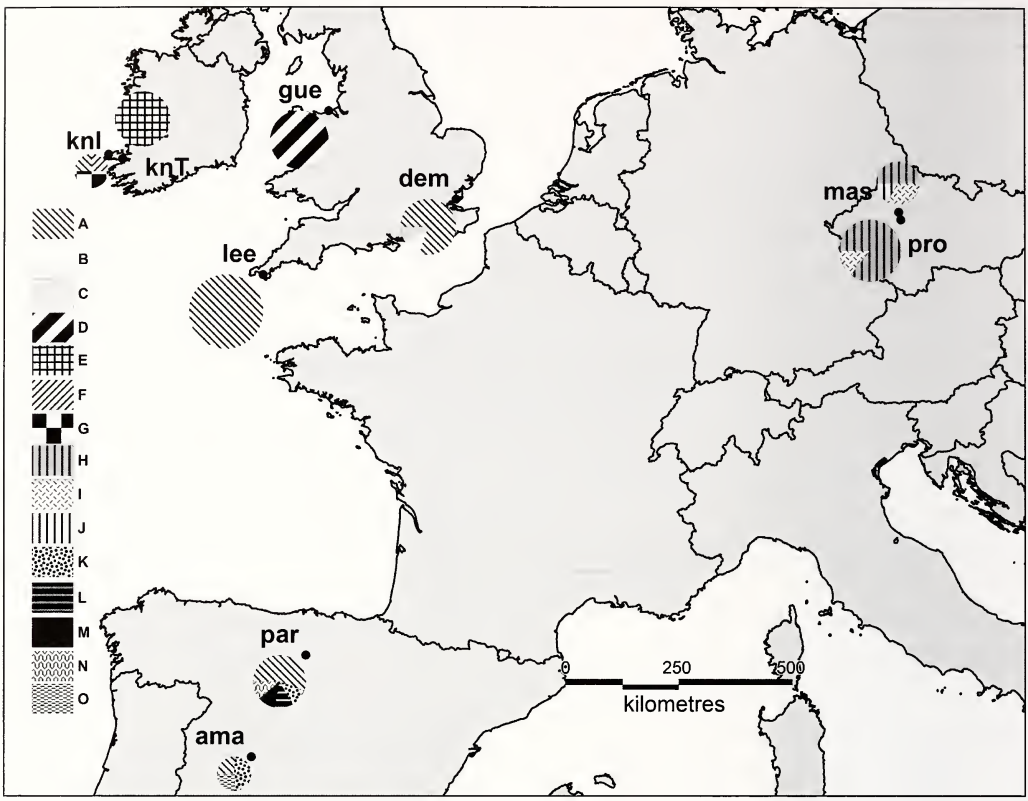

Fig. 1. Map of *Luperina nickerlii* sampling localities and haplotype structure of each sampled population based on the sequence of mitochondrial cytochrome c oxidase subunit I gene (COI). Each population is represented by a circle which is proportional to the number of specimens used for the analysis. Regions within each circle correspond to the proportion of individual COI haplotypes. Abbreviations: **dem**, *L. n. demuthi*, Strood, UK; **gue**, *L. n. gueneei*, Gronant, UK; **knI**, *L. n. knilli*, Inch, Ireland; **knT**, *L. n. knilli*, Trabeg, Ireland; **lee**, *L. n. leechi*, Loe Bar, UK; **mas**, *L. n. nickerlii*, Máslovice, Czech Republic; **pro**, *L. n. nickerlii*, Praha, Czech Republic; **par**, *L. n. albarracina* Páramos de Masa, Spain; and **ama**, *L. n. albarracina*, Amavida, Spain. Sample sizes are given in Table 2. *L. n nickerlii* also occurs elsewhere, e.g., in France and Portgual, but was not sampled there.

Genetic marker sequencing. In order to characterise the genetic distance and variability of different populations of *L. nickerlii* we selected hybrid primers for mitochondrial cytochrome c oxidase subunit I gene (COI). PCR was set up using a commercially available master mix provided by Qiagen (Hilden, Germany) containing the reagents 12.5 µl dH_2O, 2 µl 10x buffer, 2 µl $MgCl_2$, Primer F & R 2 x 1 µl, 0.4 µl l dNTP, 0.1 µl Taq polymerase, 1 µl of DNA extracts, for a total of 20 µl PCR reactions, following the manufacturer's recommendations.

An initial denaturation at 94°C for 3 min was followed by 35 cycles of 30 sec at 94°C, 30 sec at the annealing temperature of 50°C, and 1 min 30 sec at 72°C, and by a final extension step of 7 min at 72°C. Quality and amount of PCR products were checked on a 1% agarose gel. In total 1,212 bp of mitochondrial sequence were obtained. Sequences can be retrieved from GenBank under the following accession numbers: GU903504–582 and HM068967–79.

Tab. 3. Population pairwise F_{ST} values. The calculations were based on mitochondial COI sequences.

	demuthi	*gueneei*	*knilli* Inch	*knilli* Trabeg	*leechi*	*nickerlii* Máslovice	*nickerlii* Praha	*albarracina* Amavida
gueneei	0.69***	—						
knilli Inch	0.61 **	0.90**	—					
knilli Trabeg	0.77***	1.00***	0.63 NS	—				
leechi	0.13**	0.93***	0.92***	1.00***	—			
nickerlii Máslovice	0.47 ***	0.74***	0.57 *	0.74**	0.72***	—		
nickerlii Praha	0.65 ***	0.83***	0.77 **	0.85***	0.81***	0.04 NS	—	
albarracina Amavida	0.38*	0.80**	0.51*	0.80**	0.74**	0.46*	0.69***	—
albarracina Páramos	0.04 NS	0.54***	0.44**	0.63***	0.12**	0.35***	0.57***	0.10 NS

Significance level: NS = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Sequence analyses. Mitochondrial sequences were aligned using the program MUSCLE (Edgar 2004) implemented in Geneious (Biomatters Ltd., Auckland, New Zealand). Heterozygotes were detected by Heterozygote plug-in in Geneious and manually corrected. Out of 1,212 bp of mitochondrial sequence, 12 sites were informative. Neighbor-joining trees were constructed by Geneious under Jukes-Cantor genetic distance model (Jukes & Cantor 1969; Saitou & Nei 1987). Bootstrap was performed with 1,000 replicates and branches with less than 50% support were collapsed.

Population structure analysis. Analysis of Molecular Variance (AMOVA), F-statistics (fixation indices) and population pairwise differences (Excoffier et al. 1992; Weir 1996; Weir & Cockerham 1984) were calculated using Arlequin version 3.1 (Excoffier et al. 2005). Heterozygosity was computed as described in Nei (1987). Correlation of genetic and geographic distance was tested by Mantel test performed on matrices of pairwise geographic distances (given as ln km) and linearised pairwise F_{ST} values ($F_{ST}/(1-F_{ST})$) (Mantel 1967; Slatkin 1995). Hardy-Weinberg equilibrium (Guo & Thompson 1992; Levene 1949) and exact test of differentiation (Goudet et al. 1996; Raymond & Rousset 1995) were computed as implemented in Arlequin v3.1. Statistical parsimony network (Templeton et al. 1992) was constructed using TCS v1.21 (Clement et al. 2000).

Results

F_{ST} values are summarised in Tab. 3. Despite our expectations and although the habitat and phenotype of *L. n. leechi* is closest to *L. n. gueneei,* this subspecies is genetically closer to *L. n. demuthi* and *L. n. albarracina*. Pairwise F_{ST} value between *L. n. leechi* and *L. n. gueneei* was 0.93 compared to the F_{ST} between *L. n. leechi* and *L. n. demuthi* which was 0.13. High genetic differentiation was shown between *L. n. gueneei* and *L. n. knilli.*

Neighbour-joining tree based on mitochondrial marker showed only three populations as separate clusters: the population of *L. n. gueneei* was well separated (Fig. 2); *L. n. nickerlii*, *L. n. knilli* and *L. n. albarracina* individuals formed three separate

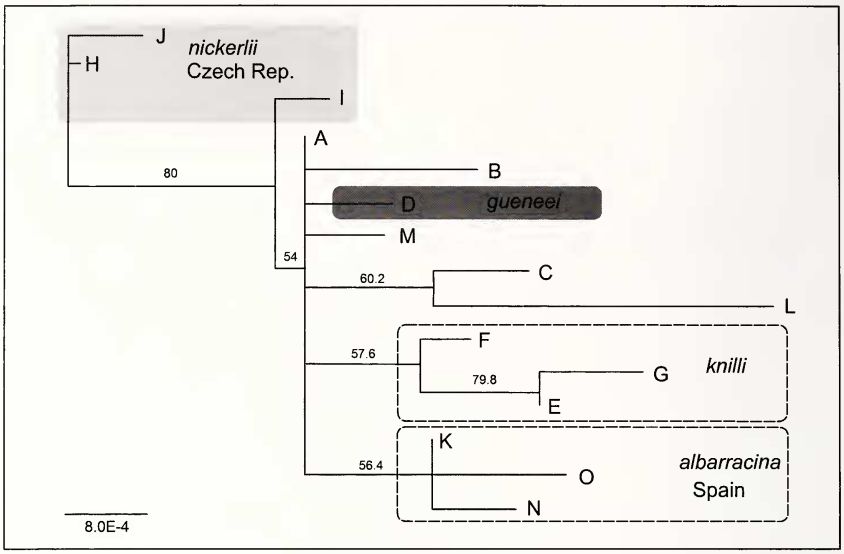

Fig. 2. Unrooted neighbor-joining tree calculated from mitochondrial COI haplotypes sampled among 91 *L. nickerlii* individuals from 9 populations. Each haplotype is represented by a letter code (for distribution and frequency see Tab. 1 and Fig. 1). Numbers above branches indicate percentage of bootstrap support out of 1,000 repetitions. Shaded rectangles mark unique haplotypes belonging to a particular population. Both *L. n. knilli* and *L. n. albarracina* (Spain) marked by an empty rectangle and dashed line contain haplotype A beside the enclosed ones.

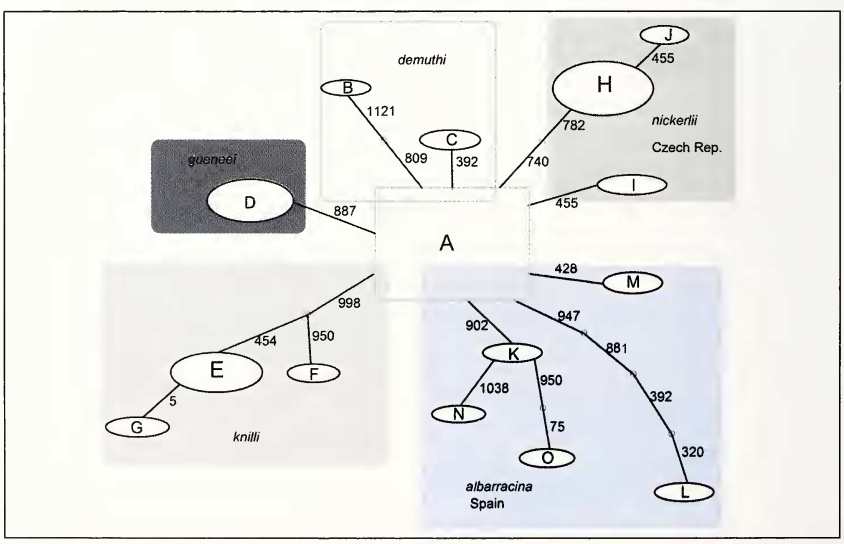

Fig. 3. Parsimony network constructed from sequence of mitochondrial cytochrome c oxidase subunit I gene (COI) of 91 *L. nickerlii* individuals from 9 populations. (*L. nickerlii* occurs elsewhere in Europe but these populations were not sampled). Total number of 15 different haplotypes were sampled (A–O listed in Tab. 2; for their prevalence see Fig. 1). Elipse areas are proportional to the haplotype frequencies. Haplotype connections were parsimonious at the 95% level. Haplotype with the highest outgroup probability is displayed as a square (A). *L. nickerlii* subspecies are marked with shaded rounded squares. Shape overlaps denote sharing of the haplotype A between different populations. Subspecies *leechi* consists of a single haplotype (A) and is not highlighted in the figure. Numbers along lines are the nucleotide positions in the sequence that changed. Empty nodes represent missing unsampled intermediate haplotypes.

Tab. 4. Genetic diversity indices based on mt COI gene.

Population	mt COI	
	Gene diversity (*h*)	Average no. of pairwise differences (π)
demuthi	0.49 +/– 0.18	1.02 +/– 0.74
gueneei	0.22 +/– 0.17	0.22 +/– 0.29
knilli Inch	0.67 +/– 0.31	1.33 +/– 1.10
knilli Trabeg	0.00 +/– 0.00	0.00 +/– 0.00
leechi	0.00 +/– 0.00	0.00 +/– 0.00
nickerlii Máslovice	0.73 +/– 0.16	1.80 +/– 1.20
nickerlii Praha	0.26 +/– 0.14	0.79 +/– 0.61
albarracina Amavida	0.83 +/– 0.22	1.50 +/– 1.12
albarracina Páramos	0.72 +/– 0.16	1.72 +/– 1.10

Tab. 5. Analysis of molecular variance (AMOVA). Mitochondrial sequence (COI) was used for the analysis. For abbreviations see legend to Fig. 1.

Population structure	Variance	% total	p	F-statistics
Each population independently				
– among populations	0.6373	63.09	0	
– within populations	0.3728	36.91		$F_{ST} = 0.6309$
Subspecies grouped				
– among groups	0.5177	49.96	0.0113	$F_{CT} = 0.4996$
– among populations within groups	0.1458	14.07	0.0432	$F_{SC} = 0.2811$
– within populations	0.3728	35.98	0	$F_{ST} = 0.6402$
(dem+lee)(gue)(knI+knT)(mas+pro)(ama+par)				
– among groups	0.6270	57.62	0.0011	$F_{CT} = 0.5762$
– among populations within groups	0.0884	8.12	0	$F_{SC} = 0.1916$
– within populations	0.3728	34.26	0	$F_{ST} = 0.6574$
(dem+lee+ama+par)(gue)(knI+knT)(mas+pro)				
– among groups	0.7286	60.54	0.0004	$F_{CT} = 0.6054$
– among populations within groups	0.1020	8.48	0	$F_{SC} = 0.2149$
– within populations	0.3728	30.98	0	$F_{ST} = 0.6902$

clusters (Fig. 1). Topology of the remaining samples was not resolved. The populations of *L. n. leechi* and *L. n. gueneei* were very homogeneous. On the other hand, high variability was revealed between populations of *L. n. albarracina* and *L. n. knilli* (Fig. 1). Values of expected heterozygosity in populations sampled are low when compared with published results in other species of Lepidoptera (Nève 2009). The estimates, however, strongly depend on the markers used (e.g., microsatellites, allozymes) and many of the studies listed by Nève (2009) have used allozymes. Among populations studied here, *L. n. leechi* showed significantly lower heterozygosity (Tab. 4). Finer structure of population genealogy was achieved by haplotype network construction (Fig. 3).

The AMOVA analysis showed the populations are structured but in contrast to our prior expectations, *L. n. leechi* is genetically closer to *L. n. demuthi*, *L. n. knilli* and *L. n. albarracina* (they widely share one haplotype) rather than to *L. n. gueneei* (Tab. 5). The Mantel test for isolation by distance was not significant ($r^2 = 0.014$; $p = 0.78$).

Discussion

We used mitochondrial markers to look at the current genetic composition of *L. nickerlii* moths in Britain and Ireland in order to understand where they came from and in particular why they are prone to splitting-off genetically identifiable groups that are isolated from other groups. Taken together the genetic markers support the null hypothesis that all the populations sampled belong to the same species *Luperina nickerlii*, despite differences in appearance and population isolation – and some differences in ecology between UK, Irish and mainland populations, although the alternative hypothesis that evolutionary divergence is too recent to be fully reflected genetically could also be considered.

More detailed analyses of the subspecies populations reveals a complex pattern of within species population differentiation. Population structure for Lepidoptera depends on the spatial distance of habitats, the dispersal abilities of the species (Nève 2009) and population origin (e.g., Hewitt 1996). *Luperina nickerlii* appears to show low dispersal abilities, perhaps due to population isolation (Spalding & Young 2011b), although occasionally singletons are found at some distance from known populations (Goater 1974; Wedd 1991), indicating dispersal activity. Results from the COI data indicate that there may have been some historical gene flow between the subspecies as isolation by distance was not supported in this study, suggesting populations were probably linked to each other in the recent past, either reflecting a collapse in a former larger pre-glacial range size or multiple post-glacial colonisation events; the degree of genetic differentiation between the British populations may suggest the second hypothesis as otherwise greater similarity between populations might be expected (e.g., Dapporto et al. 2011). Movement is likely between continental populations, e.g., in Spain and the Czech Republic, where several populations occur in close proximity, and also in Wales and south-east England where *L. n. gueneei* and *L. n. demuthi* exist in extensive dune (for *L. n. gueneei*) and saltmarsh (for *L. n. demuthi*) habitat. In contrast, the results for *L. n. knilli* indicate little interchange between populations despite occupying the same extended coastal cliff habitat in south-west Ireland and *L. n. leechi* is isolated by at least 300 km from known *L. nickerlii* populations.

The origin of the British subspecies is unclear. Those occurring on the western fringes may be part of an Atlantic Arc species assemblage that includes species such as the Quimper Snail *Elona quimperiana* (Férussac) (de Beaulieu & Le Moigne 1991) and Killarney Fern *Trichomanes speciosum* Willd (Page 1997). *L. n. leechi* shows some genetic similarity to *L. n. albarracina, L. n. knilli* and *L. n. demuthi*, but not (despite our expectations) *L. n. gueneei*. It is possible that *L. n. leechi* is a population founded by a single stray *L. n. demuthi* or *L. n. albarracina*. If so, *L. n. leechi* would be more likely to feed as larvae on *Festuca rubra* L. or *Puccinellia maritima* (Huds.) Parl. (Poaceae). However, it would appear that *L. n. leechi* may have been present on or near Loe Bar long enough to adapt to a different habitat and transfer from former food plants to *Elytrigia juncea*; in fact *Festuca rubra* is abundant in that locality. Rapid changes in larval host-plant preferences have been reported in butterflies (e.g., Asher et al. 2001; Pratt 1986–1987; Thomas et al. 2001) and moths, e.g., *Lithophane leautieri* (Boisduval, 1829) (Noctuidae) (Young 1997). Further research, perhaps involving *L. nickerlii* specimens from France and Portugal, may reveal additional linkages between the subspecies.

The origin of the extensive populations of *L. n. gueneei* remains a mystery; this subspecies appears to show significantly lower heterozygosity despite forming extensive populations on the north coast of Wales and the west coast of Lancashire, with at least some linkage between populations. Despite similarities in ecology to *L. n. leechi* (both species occurring on coastal dunes and beaches and both feeding on *Elytrigia juncea*), there is no indication that these two species have a common origin.

Genetic factors are important when assessing threatening processes and devising conservation plans for threatened species (Frankham & Ralls 1998). From a genetics perspective, the primary conservation goals are to preserve as much genetic diversity and variability as possible as well as the evolutionary processes responsible for this diversity (Clarke & O'Dwyer 2000; Coates 2000; Crandall et al. 2000). It is perhaps useful to rank populations on patch size, habitat quality and land tenure (Clarke & O'Dwyer 2000), variation in phenotype (Crandall et al. 2000) and host-plant performance (Legge et al. 1996) in addition to genetic diversity. The continental subspecies appear to have similar ecologies although there are some phenotypic and genetic differences; the British and Irish subspecies show phenotypic and genetic differences as well as having different host plants and habitats. Populations of *L. n. tardenota*, *L. n. gueneei*, *L. n. leechi* and *L. n. knilli* appear to be small and possibly declining; we provisionally suggest that key conservation effort should be directed to these subspecies.

However, not all subspecies should be considered equal (Ryder 1986). It is important to take account of the evolutionary processes associated with current levels of species diversity at the approriate geographical scale (e.g., Coates 2000). Low heterozygosity combined with increased levels of inbreeding associated with a limited number of individuals and a distorted sex ratio have been shown to decrease survival rates (e.g., Gerber 2006; Saccheri et al. 1998) and the small isolated population of *L. n. leechi* may not survive for long. The phenotypic characters that have been used to differentiate *L. n. leechi* as a subspecies are perhaps subject to environmental plasticity and may not be under genetic control. In this case this population would no longer be considered of conservation importance as *L. n. leechi* contains a single haplotype that is widely shared with other subspecies (e.g., *L. n. demuthi* and *L. n. albarracina*). The lack of genetic diversity possibly as a result of its recent origin and the small population size suggests that this isolated subspecies may be less worthy of conservation than some of the other subspecies. The case for *L. n. gueneei* is less clear as this subspecies possesses a unique haplotype and forms extensive populations on the north coasts of Wales and north-east Lancashire.

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